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Biochemical Journal Immediate Publication. Published on 25 Mar 2011 as manuscript BJ20101717

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Short title: Lu/BCAM-αII spectrin interaction

Synopsis

Lu/BCAM is a laminin 511/521 receptor, expressed in erythroid and endothelial cells, and in epithelial tissues. The RK573-574 motif of Lu/BCAM cytoplasmic domain interacts with αI-spectrin, the main component of the membrane skeleton in red blood cells. We report that Lu/BCAM binds to the non-erythroid αII-spectrin via the RK573-574 motif. Alanine substitution of this motif abolished the Lu/BCAM-spectrin interaction, enhanced Lu/BCAM half-life at the MDCK cell surface and increased Lu/BCAM-mediated cell adhesion and spreading on laminin 511/521. We showed that the Lu/BCAM-spectrin interaction mediated actin reorganization during cell adhesion and spreading on laminin 511/521. This interaction was involved in a laminin 511/521 to actin signaling pathway leading to stress fibers formation. This skeleton rearrangement was associated with an activation of the small GTP binding protein RhoA, which depended on the integrity of the Lu/BCAM laminin 511/521 binding site. It also required the Lu/BCAM-αII-spectrin interaction since its disruption decreased stress fibers formation and RhoA activation. We conclude that the Lu/BCAM-spectrin interaction is required for stress fibers formation during cell spreading on laminin 511/521 and that spectrin acts as a signal relay between laminin 511/521 and actin that is involved in actin dynamics.

Keywords : Lu/BCAM, adhesion molecule, laminin, MDCK, spectrin, cell adhesion, actin, RhoA

The abbreviations used are:

Lu	Lutheran
BCAM	basal cell adhesion molecule
RBC	Red Blood Cell
GST	glutathione-S-transferase
αR4	repeat 4 of α-spectrin
MDCK	Madin-Darby canine kidney
Ubc9	Ubiquitin enzyme conjugating 9

INTRODUCTION

Lu/BCAM glycoprotein (gp) is a member of the immunoglobulin superfamily (IgSF) that carries both the Lutheran (Lu) blood group antigens and the Basal Cell Adhesion Molecule (BCAM) tumoral antigen. It is represented by two isoforms, Lu and Lu(v13), that only differ by the length of their cytoplasmic domain (59 *vs*. 19 amino acids) [1]. The Lu gp specific cytoplasmic 40 amino acids comprise phosphorylation sites [2] and an SH3 binding motif, consistent with a receptor signaling function. Lu/BCAM represents the unique receptor for laminin 511/521 (made of α5β1γ1 and α5β2γ1 chains, respectively) [3] in normal and sickle red blood cells (RBCs) [4-6]. In human blood cells, Lu/BCAM is expressed only in erythrocytes. Lu/BCAM is also present at the basal layer of epithelia, on the surface of a subset of muscle cells, and on blood vessel endothelium [7-9]. In these tissues, Lu/BCAM is recognized as a coreceptor with integrins for laminin 511/521 [8]. Disruption of the *LU* gene in mouse provided evidence that Lu/BCAM was involved in the maintenance of normal basement membrane organization in kidney and intestine [10].

In RBCs, Lu/BCAM interacts directly with spectrin through the juxtamembrane RK573-574 residues of its cytoplasmic tail [11-13]. Identified at the inner membrane of erythrocytes, spectrins are considered as the central components of a ubiquitous and complex spectrin-actin scaffold, called the spectrin-based skeleton [14]. This network, attached to diverse cellular membranes, is involved in diverse functions including the resilience and stability of membranes, the establishment of specialized membrane domains and in vesicle trafficking. Spectrins exist as elongated flexible hetero-tetramers of 200 nm made of two α and β subunits that constitute the filaments of the network, the nodes of which are cross-linked by actin filaments. In mammals, two genes encode for α-chains (αI and αII) and five for β-chains. While αI-spectrin is the only isoform expressed in mature erythrocytes, αII-spectrin is the most common form in nucleated cells. Moreover, αI-spectrin is not expressed in epithelial cells. Each spectrin subunit is organized as an alignment of spectrin repeats, made of three α-helices each; α-spectrins contain 20 spectrin repeats. In RBCs, the Lu/BCAM binding site in αI-spectrin has been delimited to the single α4 repeat [15]. As Lu/BCAM represents a minor component of the RBCs membrane (from 1.500 to 4.000 copies/RBC), we have speculated that the Lu/BCAM-spectrin interaction might be critical for cell signaling rather than for maintenance of the membrane mechanical properties [12].

The adhesion properties of Lu/BCAM depend on its cytoplasmic domain phosphorylation, indicating an inside-out activating signal for its laminin 511/521 receptor function [2]. The physiologic stress mediator epinephrine, acting through the β2-adrenergic receptor, increases Lu/BCAM-mediated adhesion of sickle RBCs to laminin 511/521 through a protein kinase A (PKA) dependent pathway [16]. Lu/BCAM phosphorylation by PKA occurs in sickle but not in normal RBCs, concomitantly with an enhanced cell adhesion to laminin 511/521 under physiological conditions [2]. In polycythemia vera patients, who present an increased risk of thrombosis, Lu/BCAM is constitutively phosphorylated and this feature is associated with an increased RBCs adhesion to endothelial laminin 511/521 [17].

In resealed RBC ghosts, disruption of the Lu/BCAM-spectrin interaction by a spectrin peptide encompassing the α4 repeat resulted in a weakened linkage of Lu/BCAM to the spectrin-based skeleton and induced cell adhesion to laminin 511/521 [15]. Increased adhesion of RBCs from Hereditary Spherocytosis (HS) patients with a marked deficiency in spectrin, demonstrated the biological relevance of this interaction: intact HS RBCs exhibited reinforced adhesion to laminin 511/521 under physiological conditions that resulted, at least in part, from an impaired interaction between Lu/BCAM and the membrane skeleton [18]. All these observations indicate that Lu/BCAM interaction with erythroid spectrin negatively regulates cell adhesion to laminin 511/521.

In the present study, we showed that Lu/BCAM bound *in vitro* and *ex vivo* to non-erythroid αII-spectrin in kidney epithelial cells. This interaction modulated spreading and adhesion of MDCK cells to laminin 511/521. Most importantly, we showed that the Lu/BCAM-spectrin interaction was involved in a laminin 511/521 to actin signaling pathway promoting actin skeleton reorganization.

EXPERIMENTAL

The Protease Inhibitor Cocktail, Micro Spin GST Purification Module and Protein-A-Sepharose were purchased from Amersham Biosciences. Br-cAMP was from Calbiochem (Darmstadt, Germany). Primers used in Polymerase Chain Reaction and mutagenesis experiments were from MWG biotech (Ebersberg, Germany). The *in vitro* transcription and translation TNT® T7 Quick kit for PCR-amplified DNA was obtained from Promega. Dako was from DakoCytomation. Phosphatase Inhibitor Cocktail (P.I.C) for serine/threonine phosphatase, purified human laminin 511/521 mixture, fibronectin and 30% (W/V) BSA were supplied by Sigma. Lab-Tek® II Chamber Slide™ was from Nalge Nunc. Sulfo-NHS-LC-biotin and immunopure immobilized streptavidin beads were obtained from Pierce. G-LISA™ RhoA, Rac and Cdc42 Activation Assay Biochem Kit™ (absorbance Based), cell-permeable Rho Inhibitor (exoenzyme C3 transferase, CT04) were from Cytoskeleton Inc (Denver, CO). Image-iTFX signal enhancer, Calcein-AM, bis-benzimidazole Hoechst 33342, AlexaFluor® 488 Phalloidin, and NuPAGE® Novex Bis-Tris Gels were purchased from Invitrogen. Cell culture media and reagents were from Gibco BRL. Except when otherwise mentioned, reagents were purchased from Sigma-Aldrich (Saint Louis, MO, USA). 12 mm diameter, 0.4 μM pore Transwell polycarbonate filters were obtained from Costar. Millipore Electrical resistance apparatus was from Bedford. "Bio Imaging Analyser" was obtained from Fujifilm-Raytest. Evolution VF camera was from Mediacybergetics. 490 nm Multiskan RC was purchased from labsystems.

Antibodies

Antibodies directed against Lu/BCAM; monoclonal antibody (MoAb) clone F241 and the rabbit polyclonal antibody (PoAb) 602 (collaboration with Dr D. Blanchard EFS, Nantes and INTS, Paris, France). The immunopurified PoAb directed against the αII-spectrin SH3 domain was produced in our labs [19]. Goat biotinylated anti-Lu/BCAM antibody (R&D systems). Sheep anti-Human Ubc9 (A.G.Scientific Inc). AlexaFluor® 488 conjugated anti-mouse, AlexaFluor® 568 conjugated anti-rabbit antibodies (Molecular Probes, Invitrogen).

Cell lines

Human kidney carcinoma epithelial cells A498 (ATCC: HB44) were maintained in Minimal Essential Medium (MEM) Glutamax I supplemented with 10% fetal bovine serum, 100 units/ml antibiotic antimycotic, 1 mM sodium pyruvate, 0.1 mM Non Essential Amino Acids, Madin-Darby Canine Kidney cells (MDCK) (ATCC: CCL-34) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) Glutamax I supplemented with 10% fetal bovine serum, 100 units/ml antibiotic antimycotic, 0.1 mM Non-Essential amino acids. Both cell lines were grown in a humidified atmosphere at 37°C with 5% CO_2.

The full length wild-type cDNA sequence of Lu/BCAM (wt-Lu) and bearing the RK573-574AA (mt-Lu) (+1 taken as the methionine of the initiation codon) were cloned in the pcDNA3 expression vector as described [12, 20]. D343R mutation (+1 taken as the methionine of the initiation codon; the mutated residue is the same as residue 312 in reference [21] where +1 was taken as the first residue of the mature protein) was obtained by *in vitro* mutagenesis using sense primer, 5'-AGAGTGGAGGATTACCGGGCGGCAGATGACGTG-3' and antisense primer, 5'-CACGTCATCTGCCGCCCGGTAATCCTCCACTCT-3' . Stable MDCK cells expressing wt and

mutant Lu/BCAM, were obtained and amplified as described [22]. To obtain clones showing similar Lu antigen membrane expression [specific antibody binding capacity (SABC) units], cell clones were selected by serial limit dilution [22]. The SABC for wt-Lu and for mt-Lu, were 456.337 and 401.887, respectively.

Protein extraction from MDCK cells

After trypsin treatment, the pellet corresponding to 2.10^6 MDCK cells was incubated for 1 hour at 4°C in 400 µl of lysis buffer (20 mM pH 8 Tris HCl, 150 mM NaCl, 5 mM EDTA, 0.2% BSA) supplemented with 0.010 to 0.030% Triton X-100, protease inhibitor cocktail). After centrifugation (15,000 ***g***, 15 min, 4°C), the soluble cell extract (S) was loaded on a 4-12% NuPAGE® Novex Bis-Tris Gels under reducing conditions. Proteins were analyzed by Western blot using Goat biotinylated anti-Lu/BCAM antibody (0.1 µg/ml) and Sheep anti-human Ubc9 (1:3,000) as extraction control.

Co-immunoprecipitation assay

Cells (10^7) were lysed for 30 min on ice in lysis buffer (20 mM pH 8 Tris HCl, 500 mM NaCl, 2 mM $MgCl_2$, 1% Triton X-100 supplemented with protease inhibitor cocktail). Cell lysates were then sonicated and spin for 10 min at 11,000 ***g***.

Lu/BCAM were immunoprecipitated by incubating overnight at 4°C the supernatants with either anti-Lu mouse MoAb F241 or an irrelevant antibody (Moab anti-IgG1) or only with protein-A-sepharose as negative control. The beads were spin at 11,000 ***g*** for 20 min, and were washed five times with wash buffer 1 (20 mM pH 7.4 Tris HCl, 600 mM NaCl, 2 mM $MgCl_2$, 1% Triton X-100 supplemented with protease inhibitor cocktail) and twice in buffer 2 (20 mM pH 7.4 Tris HCl) to remove traces of detergent. Immunoprecipitated proteins were first eluted with glycine/HCl buffer pH 3.2 then equilibrated in Tris/HCl buffer pH 9 for 5 min incubation. Samples were loaded on a 4-12% NuPAGE® Novex Bis-Tris Gels under reducing conditions. Western blot analysis were performed using PoAb anti-αII-spectrin SH3 domain antibody (1:100,000) or Goat biotinylated anti-Lu/BCAM antibody (0.1 µg/ml).

GST–pull down assays

GST peptides were produced as previously described [12] and immobilized on Sepharose-4B glutathione beads micro spin columns. Briefly, PCR amplified cDNA fragments encoding the C-terminal ends of Lu/BCAM (residues 569 to 628), were fused with the GST protein in the pGEX-5X-3 plasmid. mt-Lu RK573-574AA was obtained by *in vitro* mutagenesis as described [12]. Spectrin repeat units corresponding to αIR3 amino acids (A253 to G384), αIIR3 (Q255 to L382), αIR4 (S361 to E486), αIIR4 (E361 to E486), αIR3-5 (A253 to L594), αIIR3-5 (Q255 to L594), αIR5 (D466 to L594), αIIR5 (L467 to L594) were obtained as a [^{35}S] Met-labeled protein with the *in vitro* transcription and translation TNT® T7 Quick kit for PCR-amplified DNA.

The products (50 µl) were incubated overnight at 4°C with GST-wt-Lu and GST-mt-Lu constructs (100 µg) immobilized on Sepharose 4B glutathione beads equilibrated in phosphate buffer saline (PBS) and antiproteases. After five washes with PBS 0.05% Tween 20, the bound proteins were solubilized in Laemmli sample buffer [23], loaded on a 4-12% NuPAGE® Novex Bis-Tris Gels, visualized and quantified by KODAK ID Image analysis software.

Confocal fluorescence microscopy and immunofluorescence of transfected cells

The transepithelial resistance was measured daily on stably transfected MDCK cells grown onto polycarbonate filters (5.10^5 cells/filter). At day 7, polarized cells were fixed 20 min with 4% paraformaldehyde, treated with 50 mM NH_4Cl in PBS, permeabilized 10 min with 0.5% Triton (PBS) and incubated with F241 anti-Lu MoAb (1:10) and the polyclonal anti-αII-spectrin SH3 domain

antibody (1:500) in Dako for 1 h at room temperature. Filters were washed with PBS-0.5% BSA and incubated with AlexaFluor® 488 and 568 conjugated anti-mouse and anti-rabbit antibodies (1:200) for 1 hr at RT, washed with PBS-0.5% BSA. Samples were examined by confocal microscopy using a NIKON EC-1 system equipped with 60 x NA 1.4 and 100 x 1.30 objectives.

Lab-Tek® II Chamber Slide™ were coated with 2 µg/cm^2 of either laminin 511/521 or fibronectin overnight at 4°C (in 400 µl). Wells were then washed twice with PBS and were subsequently coated with 1% BSA at 37°C for 1 hour before two additional washes. 10^4 mock-transfected MDCK cells or transfected cells expressing wt-Lu, mt-Lu, D343R Lu mutant or Lu(v13) were added to the wells (in 400 µl). Cells were incubated in in serum-free medium for 90 min at 37°C in presence or absence of 1 µg/ml per well of the cell-penetrating form of the *Clostridium botulinum* C3 toxin, an ADP ribosyltransferase that selectively ribosylates Rho proteins, rendering them inactive After this incubation time, cells were fixed 20 min with 4% paraformaldehyde, treated with 50 mM NH_4Cl in PBS, permeabilized 10 min with 0.5% Triton (PBS), saturated 30 min in Image-iTFX signal enhancer solution. Cells were washed with PBS-0.5% BSA and incubated with Phalloidin AlexaFluor® 488 (1:50) for 1 h at RT. Samples were examined by confocal microscopy.

Cell surface biotinylation assay

Polarized MDCK monolayers expressing either wt-Lu or mt-Lu were cultured on filters as described above. Newly synthesized proteins were labeled by adding 150 µCi [^{35}S] methionine/[^{35}S] cysteine in the cell culture medium for 20 min incubation at 37°C. After washing with complete medium, cells were incubated in non-radioactive medium for 60, 90, 120, 150 and 180 min at 37°C. After each incubation time, cells were washed twice with cold PBS and incubated at the basolateral side with 0.5 mg/ml Sulfo-NHS-LC-biotin. After immunoprecipitation of total Lu/BCAM, half of the eluted proteins were diluted with Laemmli 3X buffer (total Lu /BCAM) and the other half was incubated in 500 µl of lysis buffer for 3 h with immunopure immobilized streptavidin beads to isolate membrane Lu/BCAM. Beads were washed three times with lysis buffer and Lu/BCAM was eluted in 20 µl of Laemmli buffer for 5 min at 100°C. Eluates from both steps (total and membrane Lu/BCAM) were analyzed by SDS-PAGE on 8% polyacrylamide gel under reducing conditions followed by Western blot probed with rabbit polyclonal anti-Lu antibody 602 (1:5000). Samples of biotinylated Lu proteins were analyzed using a "Bio Imaging Analyser" to quantify the newly delivered membrane proteins as a function of time.

Cell-spreading assay

The 12-wells plates were coated with either 2 µg/cm2 of laminin 511/521 or 1% BSA overnight at 4°C. Wells were washed twice with PBS and were subsequently coated with 1%BSA at 37°C for 1 h before two additional washes. MDCK cells were washed twice and added to the wells (10^5 per wells) in serum-free medium. After different incubations time (30, 60 and 90 min) at 37°C, spread and round cells were quantified in four representative areas by microscopy (Leitz, x100) using a computerized image analysis system (Biocom VisioL@b 2000). The counted cells were then averaged and presented as mean percentage of spread cells. A *p* value greater than 0.05 was considered not significantly different.

Cell matrix adhesion assay

2.10^6 mock-transfected MDCK cells or transfected wt-Lu or mt-Lu cells were labeled with 5 µM calcein-AM when 2.10^6 MDCK -mt-Lu control cells were labeled with 1 µg/ml Hoechst 33342, 30 min at 37°C/5% CO_2, then cells were washed twice with prewarmed serum-free medium. MDCK, wt-Lu or mt-Lu cells were then mixed with control cells as internal standard in a 1/1 ratio. 1 ml of the mixed cells was then put in laminin 511/521 pre-coated 12 well plates. After 90 min incubation at 37°C/5%

CO_2, cells were washed twice with prewarmed serum-free medium to remove unattached cells and visualized by fluorescence using Evolution VF camera. Ten images were acquired for each sample, and adherent cells were count using Image-Pro® Plus software. Results are expressed as the mean percentage of adherent transfected cells versus control adherent cells.

Phosphorylation assays in transfected cells

MDCK-wt-Lu and mt-Lu (5.10^6) growth in in 6-wells plates were washed three times in DMEM without phosphate and incubated in the same medium for 2 h at 37°C. Cells were then incubated with 300 µCi of orthophosphate ^{32}P (25 mM) for 90 min at 37°C and one well with wt-Lu or mt-Lu cells was supplemented with 1X of P.I.C (90 min) or 1 mM br-cAMP (30 min). Cells were washed twice with ice cold PBS and incubated in lysis buffer (20 mM pH 8 Tris HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.2% BSA, supplemented with protease inhibitor cocktail and 1X PIC) for 1 h at 4°C. Immunoprecipitation of Lu/BCAM was describing above (Co-immunoprecipitation assay). The beads were washed five times in lysis buffer. Immunoprecipitated proteins were first eluted 5 min at 100°C with 3X laemmli supplemented with 5% beta-mercapto-ethanol and then loaded on a 4-12% NuPAGE® Novex Bis-Tris Gels under reducing conditions. Analyzed was performed by Western blot using Goat biotinylated anti-Lu/BCAM antibody (0.1 µg/ml) or using the "Bio Imaging Analyser".

Rho family proteins activation and inhibition assay

The 6-wells plates were coated with laminin 511/521 or fibronectin as described before. 25.10^4 mock-transfected MDCK cells and wt-Lu or mt-Lu cells were added to the wells for 90 min. Active Rac1, 2, 3 and RhoA were measured using the G-LISA™ Rac or RhoA or Cdc42 Activation Assay Biochem Kit™ according to the manufacturer's instructions. GTP Rac, RhoA and Cdc42 were used as positive control. The absorbance signals were detected at 490 nm.

Statistical analysis

Results are expressed as mean. Data set were analyzed by the Mann-Whitney non parametric test to determine statistical significance between mt-Lu and wt-Lu. Statistical significance is defined as $p<0.05$.

RESULTS

Lu/BCAM interacts with αII-spectrin in non-erythroid cells via its RK573-574 residues

We investigated the potential interaction of Lu /BCAM with the αII-spectrin isoform expressed in non-erythroid cells. Immunoprecipitation (IP) assays were performed using two cell lines expressing endogenous Lu/BCAM: the human kidney epithelial cells A498 and the human microvascular endothelial cells HMEC-1. After Lu/BCAM IP, Western blotting (WB) analysis revealed the presence of αII-spectrin indicating that it was co-immunoprecipitated with Lu /BCAM from A498 cells (Fig. 1A, left panel) and HMEC-1 cells (data not shown), and suggesting an interaction between these two proteins. Neither spectrin (270 kDa) nor Lu/BCAM (90 kDa) were detected when an irrelevant antibody (IgG1) or Protein A-Sepharose (PA) alone were used for IP. The RK573-574 residues of the cytoplasmic tail of Lu/BCAM are required for binding to αI-spectrin in RBCs [12]. The involvement of these residues in the interaction with αII-spectrin was also investigated by IP using transfected MDCK cells expressing either wild-type Lu/BCAM (wt-Lu) or the RK573-574AA Lu/BCAM mutant (mt-Lu). As expected, αII-spectrin was co-immunoprecipitated with wt-Lu (Fig. 1A, right panel). Faint quantities of αII-spectrin, similar to those obtained with the IgG1 negative control, were detected from cells expressing mt-Lu (Fig. 1A, right panel), indicating that the RK573-574AA mutation abolished the Lu/BCAM-αII-spectrin interaction. These results indicated that endogenous and recombinant Lu/BCAM interacted with αII-spectrin in both epithelial and endothelial cells; this interaction involved the Lu /BCAM cytoplasmic RK573-574 motif.

Lu/BCAM binds to α4 repeat of αII-spectrin

The Lu/BCAM cytoplasmic domain binds to αI-spectrin α4 repeat (α4R) in RBCs [15]. Sequence analyses revealed that the αII-spectrin α4R (residues E361 to E486) shares 57% of identity with its αI-spectrin corresponding repeat. We tested the involvement of the αII-spectrin α4R in the interaction with Lu/BCAM using GST pull down assays. α3R, α4R, α5R and α3->5R were produced as [^{35}S] Met-labeled peptides by *in vitro* transcription and translation (TNT products) (Fig. 1B), and their binding activity to the cytoplasmic domain of Lu/BCAM, produced as a GST-fusion protein, was analyzed. As shown in Fig. 1B, the spectrin peptides encompassing either the α3->5R or only the α4R were pulled down with the wt-Lu cytoplasmic tail (GST-wt-Lu), giving a signal 3- to 5-fold higher than those obtained with either GST alone or the mt-Lu cytoplasmic domain (GST-mt-Lu). In contrast, the pull-down signals obtained with the α3R and α5R were similar for GST, GST-wt-Lu and GST-mt-Lu. These results indicated that αII-spectrin α4R was involved in the interaction with the Lu/BCAM RK573-574 motif.

Disruption of the Lu/BCAM-spectrin interaction increases Lu/BCAM extractability in epithelial cells

To verify the involvement of the spectrin- Lu/BCAM interaction as a link between Lu/BCAM and the membrane cytoskeleton, we examined the detergent solubility of recombinant wt-Lu and mt-Lu in epithelial MDCK cells. Cells were treated with increasing concentrations of Triton X-100 (0.01 to 0.1%) and the amount of solubilized Lu/BCAM was analyzed by WB using the Ubiquitin-conjugating enzyme 9 (Ubc9) (20 kDa) as a cytoplasmic marker and loading control. As shown in Fig. 1C, wt-Lu was not detected in the soluble fraction below 0.025% of Triton X-100. Conversely, mt-Lu was extracted starting from 0.01% of Triton X-100 indicating that disruption of the Lu/BCAM-spectrin interaction increases the cytoskeleton-unbound Lu/BCAM fraction in non-erythroid cells.

Interaction with spectrin is not involved in the membrane targeting of Lu/BCAM

The basolateral expression of Lu/BCAM in polarized epithelial cells depends on the integrity of a dileucine motif at position 608-609 of the cytoplasmic domain [24]. To determine the potential role of αII-spectrin in Lu/BCAM membrane targeting, confluent monolayers of MDCK clones expressing similar amounts of either wt-Lu (400,000 molecules/cell) or mt-Lu (450,000 molecules/cell) were grown on filters, then labeled with both monoclonal anti-Lu/BCAM and polyclonal anti-αII-spectrin antibodies. Confocal microscopy analysis showed that both cell types exhibited a polarized phenotype and form tight junctions at confluence, as indicated by daily transepithelial resistance measurements (900 ohms/well after 7 days of culture). As shown in Fig. S1, spectrin (*red*) and wt-Lu (*green*) were expressed at the cell membrane and displayed a lateral expression, as expected. The merge panels (*yellow*) indicated that wt-Lu and spectrin were in the same lateral membrane compartment. The same pictures were obtained with MDCK cells expressing mt-Lu, indicating that disruption of the Lu/BCAM-spectrin interaction did not modify the Lu/BCAM membrane expression pattern. Therefore this interaction is not required for the correct lateral targeting of Lu/BCAM in polarized epithelial cells.

Interaction with αII-spectrin plays a role in the stability of Lu/BCAM at the epithelial cell membrane

As the Lu/BCAM-spectrin interaction was not involved in the cell surface localization of Lu/BCAM, we investigated the putative role of this interaction in Lu/BCAM membrane delivery and turn-over using pulse-chase experiments combined with surface protein biotinylation.

As the newly-delivered Lu/BCAM at the membrane has been reported to reach a peak between 60 and 90 min of chase [24], the chase experiment on polarized MDCK cells was started at 60 min after the radiolabeling step and followed up to 180 min. Total cell surface proteins were labeled with biotin before cell lysis and IP. Total Lu/BCAM was immunoprecipitated and the biotinylated fraction, corresponding to Lu/BCAM expressed at the cell membrane, was purified by streptavidin-agarose beads. The amounts of radiolabeled Lu/BCAM presents on the membrane after 60, 90, 120, 150 and 180 min were determined by SDS-PAGE of biotinylated proteins followed by autoradiography. Newly-synthesized wt-Lu and mt-Lu were correctly addressed to the lateral membrane as both were present at the cell surface after 60 min of chase (Fig. 2A). This result confirmed that the interaction with spectrin was not involved in Lu/BCAM membrane targeting. However, the turn-over of mt-Lu and wt-Lu were significantly different as shown by the extended mt-Lu half-life at the membrane. The newly delivered amounts of mt-Lu at 60 min remained unchanged up to 180 min while wt-Lu decreased soon after 60 min. This difference in kinetics was not due to protein degradation as WB showed that equivalent amounts of each protein were immunoprecipitated for all chase times (Fig. 2B).

To quantify the turn-over of both proteins, the intensity of all bands was determined and the ratio “radiolabeled /biotinylated” was calculated. As shown in Fig. 2C, radiolabeled wt-Lu decreased at the membrane over time with almost half of the proteins being internalized after 90 min as compared to 60 min. Conversely, mt-Lu showed an enhanced stable membrane expression as newly delivered mt-Lu did not undergo significant internalization up to 180 min of chase, indicating that the lack of interaction with spectrin decreases the membrane turn-over of Lu/BCAM in polarized MDCK cells.

Interaction of Lu/BCAM with spectrin modulates spreading and adhesion of epithelial cells to laminin 511/521

Alteration of the spectrin-based skeleton as well as disruption of the Lu/BCAM-spectrin interaction resulted in an enhanced Lu/BCAM-mediated adhesion of RBCs and K562 cells to laminin 511/521 [15, 18]. To investigate whether the interaction with spectrin could similarly modulate Lu/BCAM-mediated adhesion of epithelial cells, we analyzed the spreading and adhesion to laminin 511/521 of MDCK cells expressing either wt-Lu or mt-Lu as described under “Experimental” (Fig. 3A).

We first compared the ability of MDCK cells to spread on laminin 511/521 and BSA, used as negative control. Ninety minutes after plating, only 12% and 14% of mock-transfected MDCK cells could spread on BSA and laminin 511/521, respectively (Fig. 3B). MDCK cells expressing either wt-Lu or mt-Lu presented a similar behavior on BSA but exhibited 38% and 60% of spread cells on laminin 511/521 at 90 min, respectively (Fig. 3B). Cell spreading on laminin 511/521 was higher for mt-Lu than for wt-Lu expressing cells and was observed as soon as 30 min after plating (29% vs 14%).

Cell adhesion to laminin 511/521 was also investigated. As expected, Lu/BCAM expression increased cell adhesion to laminin 511/521 as evaluated by the percentage of adherent cells: 65% for wt-Lu vs 20% for mock-transfected MDCK cells (Fig. 3C). Cell adhesion to laminin 511/521 was particularly reinforced for mt-Lu expressing cells with 95% of adherent cells. These results indicated that disruption of the Lu/BCAM-spectrin interaction significantly increased Lu/BCAM-mediated epithelial cell adhesion to laminin 511/521.

The reinforced adhesion of mt-Lu expressing MDCK cells is not related to the Lu/BCAM phosphorylation status

The increased adhesion of pathological red cells to laminin 511/521 is associated with PKA-mediated Lu/BCAM phosphorylation [2, 17]. As disruption of the Lu/BCAM-spectrin interaction reinforced the MDCK cell adhesion to laminin 511/521, we investigated whether this feature was related to a PKA-stimulated Lu/BCAM phosphorylation. Phosphorylation assays were performed using MDCK cells expressing either wt-Lu or mt-Lu. Lu/BCAM was immunoprecipitated from ^{32}P radiolabeled cells in the presence of a serine/threonine phosphatases inhibitor cocktail (PIC), or of br-cAMP, a stable membrane permeable cAMP analog that activates PKA.

In the absence of activators, and 90 min after cell plating on laminin 511/521, both wt-Lu and mt-Lu presented a similar basal phosphorylation level, as shown by autoradiography and WB (Fig. 4, compare lanes 1 and 4). The amount of immunoprecipitated Lu/BCAM was estimated by WB and Lu/BCAM phosphorylation was normalized by calculating the ratio of the phosphorylation signal and the WB intensity of each band. The phosphorylation level of both proteins was similarly induced in the presence of PIC and br-cAMP (Fig. 4), indicating that disruption of the Lu/BCAM-spectrin interaction did not alter the Lu/BCAM phosphorylation status.

Lu/BCAM-spectrin interaction is required for stress fibers formation on laminin 511/521

As cell adhesion and motility require actin rearrangements, we tested whether the Lu /BCAM-spectrin interaction could induce specific actin reorganization during MDCK cell adhesion and spreading. Low density plated cells were stained with phalloidin to label F-actin, 90 min after seeding on either laminin 511/521 (Fig. 5a-e) or fibronectin (Fig. 5f-j). Analyses were performed by confocal microscopy focusing on the cell basal membrane. In mock transfected cells, actin was detected as peripheral spike-like protrusions and cortical ring as well as cytoplasmic punctuated forms on both laminin 511/521 and fibronectin substrates (Fig. 5a,f). The same actin expression pattern was observed in all wt-Lu expressing cells plated on fibronectin (Fig. 5g). When these cells were plated on laminin 511/521, they exhibited a better spreading with extended lamellipodia. An increase in actin stress fibers was observed in 46% of Lu/BCAM expressing cells (Fig. 5b). To determine the putative involvement of the Lu/BCAM-laminin 511/521 interaction in the stress fibers formation, MDCK cells expressing the D343R Lu/BCAM mutant, which is unable to bind to laminin 511/521 (the mutated residue is the same as residue 312 in reference [21]), were analyzed. These cells did not form stress fibers neither on laminin 511/521 nor on fibronectin (Fig. 5c,h). Absence of stress fibers was also observed for MDCK cells expressing mt-Lu, plated on laminin 511/521 or fibronectin (Fig. 5d,i). These results indicated that the stress fibers formation in MDCK cells plated on laminin 511/521 was induced by the Lu/BCAM-laminin 511/521 interaction and that the Lu/BCAM-spectrin interaction was involved in a laminin

511/521 to actin signaling pathway leading to actin reorganization.

To determine the putative role of the 40 C-terminal amino acids of Lu gp in regulating stress fibers formation, the same experiment was performed using MDCK cells expressing the short isoform Lu (v13). This isoform is truncated for the 40 C-terminal amino acids comprising the proline-rich SH3-binding domain, the dileucine motif, and the phosphorylation sites. Similarly to wt-Lu expressing cells, 64% of cells expressing Lu(v13) were able to form stress fibers when plated on laminin 511/521 (Fig. 5e), indicating that Lu/BCAM-mediated actin reorganization was independent of its proline-rich SH3-binding domain and of potential signaling events that could be mediated by its cytoplasmic tail.

Laminin 511/521 activates a RhoA-dependent signaling pathway via the Lu/BCAM-spectrin interaction

Rho GTPases are important regulators of the actin cytoskeleton and are involved in cell shape and motility. The activation of the small GTP-binding protein RhoA plays a role in stress fibers formation whereas Rac and Cdc42 are responsible for the actin polymerization in lamellipodia [25-27]. We compared RhoA, Rac and Cdc42 activities in mock-transfected MDCK cells and in cells expressing either wt-Lu or mt-Lu. Activities were evaluated from equal amounts of lysates obtained from cells plated for 90 min on either laminin 511/521 or fibronectin (Fig. 6). No changes could be observed in Rac and Cdc42 activity for all conditions (Fig. 6B,C). In contrast, cells expressing wt-Lu and plated on laminin 511/521 exhibited a significant increase of RhoA activity as compared to mock and mt-Lu cells (Fig. 6A). These data suggested the involvement of RhoA activation in the actin reorganization associated with the Lu/BCAM-spectrin interaction.

To further investigate the involvement of the RhoA pathway, we used the exoenzyme C3 transferase as a RhoA inhibitor. Pretreatment with the C3 transferase for 2 hours is sufficient to inhibit the stress fibers formation in wt-Lu and Lu(v13) MDCK cells plated on laminin 511/521 indicating that RhoA is necessary for the specific Lu/BCAM induced actin reorganization (Fig.7).

DISCUSSION

Lu/BCAM interacts with spectrin in non-erythroid cells.

This study identified the αII-spectrin isoform as a direct cytoplasmic partner of Lu/BCAM *in vitro* and *ex vivo* in different cellular contexts. The co-immunoprecipitation of endogenous spectrin with Lu/BCAM from kidney epithelial A498 cells gave the physiological relevance of the GST pull-down interactions.

Positively charged juxta-membrane amino acids in adhesion molecules, such as CD44 and ICAM-2, were shown to interact with cytoskeletal protein 4.1 and ERM proteins (ezrin, radixin, moesin) [28]. As in RBCs, Lu/BCAM RK573-574 motif interacted directly with αII-spectrin α4R and it could be assumed that this interaction might occur in all cell types where these two proteins are expressed. In humans, αI and αII-spectrins share 55% of identity in the amino acid sequence. α-spectrins are composed of 20 repeats and αII-spectrin α4R exhibits 57% of identity with its corresponding erythroid αI-spectrin repeat. Moreover, Lu/BCAM RK573-574 motif and surrounding sequences are conserved in mammals.

Effects of Lu/BCAM-spectrin interaction on Lu/BCAM membrane localization and stability

Lu/BCAM is endogenously expressed in epithelial kidney A498 cells at 70,000 copies /cell [22]. Its lateral expression in polarized epithelial cells depends on the dileucine motif of its cytoplasmic domain [4].

Moreover, recruitment of Lu/BCAM at the lateral surface in epithelial cells, adjacent to laminin α5-containing extracellular matrix, suggests a role for laminin 511/521 in its membrane localization

[8]. Indeed, Lu/BCAM expression was dramatically reduced in various tissues of mouse embryos lacking laminin α5 chain, while it was significantly increased in the heart of transgenic mice overexpressing this chain [29, 30].

Our results pointed out the predominance of the dileucine motif for Lu/BCAM lateral targeting. Our immunostaining experiments indicated that interaction with spectrin was not required for the proper targeting of Lu/BCAM in polarized epithelial MDCK cells.

In conclusion, the RK motif does not appear to be involved in Lu/BCAM membrane targeting, but this interaction could play a role in Lu/BCAM membrane turnover. In the pulse-chase experiments, we observed that Lu RK573-574AA mutant had an extended half-life at the cell surface. Since αII-spectrin interacts with syntaxin family proteins, which play a key role in the vesicle fusion during exocytosis and endocytosis [31], our results suggest that spectrin might be involved in Lu/BCAM turnover through the endocytic pathway.

Role of the Lu/BCAM-spectrin interaction during cell spreading and adhesion.

It was hypothesized that the Lu/BCAM-spectrin interaction might be critical for signaling and laminin 511/521 receptor function rather than in the maintenance of the RBC shape. Lu/BCAM is involved in the abnormal adhesion of red cells to laminin 511/521 and endothelium in pathological situations [2, 5, 16, 17]. We and others have demonstrated that the interaction of Lu/BCAM with erythroid spectrin negatively regulated its adhesive receptor function in normal and spectrin-deficient HS RBCs [15, 18]. In non-erythroid adherent cells, partial αII-spectrin depletion was associated with loss of cell spreading, defective adhesion and decrease and irregularity of focal adhesion points [32].

Our results showed that Lu/BCAM plays a critical role during epithelial cell adhesion to laminin 511/521. Cell adhesion and spreading to laminin 511/521 were significantly reinforced in cells expressing Lu RK573-574AA mutant which could be a consequence of its extended half-life at the cell surface. Other proteins of the spectrin-based cytoskeleton, like the adapter protein ankyrin G, can also modulate adhesion molecules' functions including cell adhesion, migration and membrane distribution. The membrane expression of L1CAM, involved in the migration of neuronal growth cones and the static adhesion between adjacent axons, is regulated by its interaction with ankyrin G. Inhibition of this interaction led to retrograde movements of L1CAM in the cell membrane and stimulated L1CAM-mediated neurite outgrowth [33].

To date, the Lu/BCAM laminin 511/521 receptor function was known to be activated by two pathways in pathological RBCs: one involving the phosphorylation of its cytoplasmic domain by PKA [2]; and the other mediated by Lu/BCAM cytoplasmic domain interaction with the spectrin-based cytoskeleton [15, 18]. Our present study indicates that interaction between Lu/BCAM and spectrin regulates epithelial cell adhesion and spreading to laminin 511/521. This regulation is not related to an increased Lu/BCAM phosphorylation of Lu RK573-574AA mutant. Conversely, the laminin 511/521 binding could induce Lu/BCAM cytoplasmic tail phosphorylation, thus modulating its interaction with spectrin. Further experiments should be done to investigate this question.

Consequences on actin dynamics.

Cell adhesion to extracellular matrix triggers outside-in signaling pathways leading to actin skeleton reorganization with extension of lamellipodia and filopodia. Here, we showed that Lu/BCAM induced stress fibers formation during the early steps of cell adhesion and spreading to laminin 511/521. These changes were due to the Lu/BCAM-laminin 511/521 interaction as cells expressing the D343R mutant, which is defective for laminin 511/521 binding, were unable to induce stress fibers formation. This rearrangement of the actin skeleton was associated with an activation of the small GTP-binding protein RhoA which is known to regulate actin polymerization and stress fibers formation [27]. Similarly, RhoA activation was only observed in cells expressing wt Lu/BCAM. We can conclude that Lu/BCAM binding to laminin 511/521 drives actin filament formation.

The involvement of laminin α5 in cell spreading and in polymerization of parallel actin filaments, named filipodia-like microspikes, has been also demonstrated in primary dental epithelial cells in which laminin α5 interacts with α6β4 integrins. This interaction participates in the activation of phosphatidylinositol 3 kinase-Cdc42/Rac pathways [34]. We show that Cdc42/Rac pathways are not involved in the Lu/BCAM induced actin reorganization.

The signaling pathway induced by Lu/BCAM binding to laminin 511/521 and leading to actin reorganization requires the Lu/BCAM-αII-spectrin interaction. Disruption of this interaction reduced both the stress fibers formation and the RhoA activation. We showed that both Lu/BCAM isoforms acted similarly regarding actin reorganization. The ability of the short isoform Lu(v13) to induce stress fibers formation indicated that this was independent of the proline-rich SH3-binding domain and the phosphorylation sites present in the long isoform 40 C-terminal amino acids. Our results strongly suggest that the signaling cascade leading to actin reorganization upon Lu/BCAM binding to laminin 511/521 is vehicled by spectrin. All these results provide new insights into a novel signaling pathway regulating the actin cytoskeleton via the Lu/BCAM-spectrin interaction that links the upstream laminin 511/521 binding signal to the downstream RhoA activation.

The role of αII-spectrin in actin organization has been recently demonstrated, as αII-spectrin knocked-down melanoma WM-266 cells exhibited modifications of the actin cytoskeleton such as a loss of stress fibers [32]. αII-Spectrin, via its SH3 domain, has been also implicated in initiating Rac activation in the specialized β3 integrin clusters that initiate cell adhesion and spreading [35]. Moreover, the SH3 domain of αII-spectrin was demonstrated to bind proteins involved in the regulation of actin cytoskeleton dynamics such as two members of the Mena-VASP family (EVL, VASP) and Tes [36-39]. VASP participates in actin fiber formation and the αII-spectrin-VASP complexes regulate cortical actin cytoskeleton assembly with implications in cell–cell contact formation. Overexpression of TES resulted in increased cell spreading and decreased cell motility whereas Tes knock-down in HeLa cells resulted in loss of actin stress fibers and reduced RhoA activity [40]. All these data reinforce the idea of a pivotal role of spectrin in actin-dependent processes.

In conclusion, spectrin is involved in regulating Lu/BCAM function and expression in non-erythroid cells. Our findings provide new evidence that spectrin plays a novel role as a linker between extracellular signals, triggered by laminin 511/521, and intracellular events modulating actin dynamics.

ACKNOWLEDGEMENTS

*We are grateful to Julien Picot for helpful advice on Flow cytometry experiments.

Founding

This investigation was supported in part by the Institut National de la Transfusion Sanguine (INTS), the Institut National de la Santé et de la Recherche Médicale (INSERM) and Université Paris Diderot – Paris7.

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FIGURE LEGENDS

Fig.1. (A) Co-immunoprecipitation of αII-spectrin with Lu/BCAM in A498 and MDCK epithelial cells. Lysates from A498 cells expressing both endogenous spectrin and Lu/BCAM, and MDCK cells expressing recombinant wt-Lu or mt-Lu proteins were treated with either the mouse monoclonal anti-Lu/BCAM antibody F241 (Lu), or mouse irrelevant antibody (IgG1) or Protein A Sepharose (PA). Presence of spectrin (270 kDa) and Lu/BCAM (90 kDa) in eluates was detected by Western blot (WB) using a PoAb against the αII-spectrin SH3 domain and a biotinylated goat anti-Lu/BCAM antibody, respectively. **(B) Binding assay of αII-spectrin peptides to Lu/BCAM**. [^{35}S]-labeled αII-spectrin peptides corresponding to α3R, α4R, α5R and α3->5R spectrin repeats (TNT products) were incubated with GST, GST-wt-Lu and GST-mt-Lu immobilized on Sepharose 4B glutathione beads. After extensive washing the bound proteins were eluted in Laemmli sample buffer, submitted to electrophoresis and visualized by autoradiography. Spectrin repeats monomers (*) and dimers (**). **(C) Triton extractability of Lu/BCAM protein in MDCK cells**. MDCK cells expressing either recombinant wt-Lu or mt-Lu protein were lysed in the presence of increasing concentrations of Triton X-100. Extracted proteins were analyzed by Western blot using anti-Lu/BCAM and anti-Ubc9 antibodies. The 20 kDa endogenous cytoplasmic enzyme Ubc9 was used as extraction control.

Fig.2. The expression level and the turnover of Lu/BCAM protein at the membrane of polarized MDCK cells. MDCK cells expressing wt-Lu or mt-Lu were grown on transwell filters. (A) Newly-delivered wt-Lu and mt-Lu proteins at the cell surface as determined by autoradiography of biotinylated Lu/BCAM. (B) Western blot showing total amount of wt-Lu or mt-Lu expressed at the membrane at each chase time, (C) The curves show the ratio of "radiolabeled biotinylated Lu/total biotinylated Lu" at the membrane, reflecting the turn-over of newly-delivered wt-Lu (▲) or mt-Lu (■) proteins at the cell surface as a function of time. The curves represent means of 3 experiments.

Fig.3. Adhesion of MDCK cells to laminin 511/521. Mock, wt-Lu or mt-Lu MDCK cells were incubated at 37°C for 30, 60 and 90 min in wells coated with either 2 μg/cm^2 of laminin 511/521 or 1% BSA. (A) Photos showing cell morphology of each cell line at 90 min. (B) The histogram indicates the percentage of spread cells after 30, 60 and 90 min of adhesion to laminin 511/521 or to BSA. (C) The histogram indicates the percentage of adherent cells after 90 min on laminin 511/521. Each result represents the mean for 8 experiments in duplicates. Statistical significance was determined using Mann-Whitney test ($p < 0.05$).

Fig.4. Phosphorylation of Lu/BCAM protein in MDCK cells. (A) MDCK-wt-Lu and -mt-Lu cells were incubated for 90 min at 37°C with orthophosphate ^{32}P in the absence or presence of PIC (lanes 2 and 5) or br-cAMP (lanes 3 and 6). After immunoprecipitation, radiolabeled Lu/BCAM was analyzed by autoradiography. Western blot (WB) analysis of immunoprecipitated products was performed using Goat biotinylated anti-Lu/BCAM antibody. The P/WB ratio represents the proportion of phosphorylated Lu/BCAM in total immunoprecipitated Lu/BCAM proteins.

Fig.5. Immunofluorescence studies of actin in adherent MDCK cells. 10^5 mock MDCK cells (a,f), MDCK-wt-Lu (b,g), -D343R-Lu (c,h), -mt-Lu (d,i) or Lu(v13) (e,j) cells were plated for 90 min into wells coated with 2 μg/cm^2 of laminin 511/521 (left column) or fibronectin (right column). Imaging shows F-actin labeled with phalloidin. Bars = 10 μM.

Fig.6. RhoA and Rac 1, 2, 3 and Cdc42 activity assays. The active forms of RhoA, Rac, and Cdc42 were colorimetrically detected using G-LISA kit. (A) RhoA activity (B) Rac1, 2, 3 activities and (C) Cdc42 in MDCK cells plated for 90 min on either laminin 511/521 or fibronectin. Each result represents the mean of 3 assays performed in duplicates. Statistical significance was determined using Mann-Whitney test ($p < 0.05$).

Fig.7. Effects of Exoenzyme C3 Transferase on the RhoA-dependent signaling pathway via the Lu/BCAM-spectrin interaction induced stress fibers formation
10^5 MDCK-wt-Lu, -Lu (v13) and -mt-Lu cells were plated for 90 min into wells coated with 2 µg/cm^2 of laminin 511/521 and were untreated (left column) or treated with 1.0 µg/ml of C3 toxin (right column). Imaging shows F-actin labeled with phalloidin. Bars = 10 µM.

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Fig.1

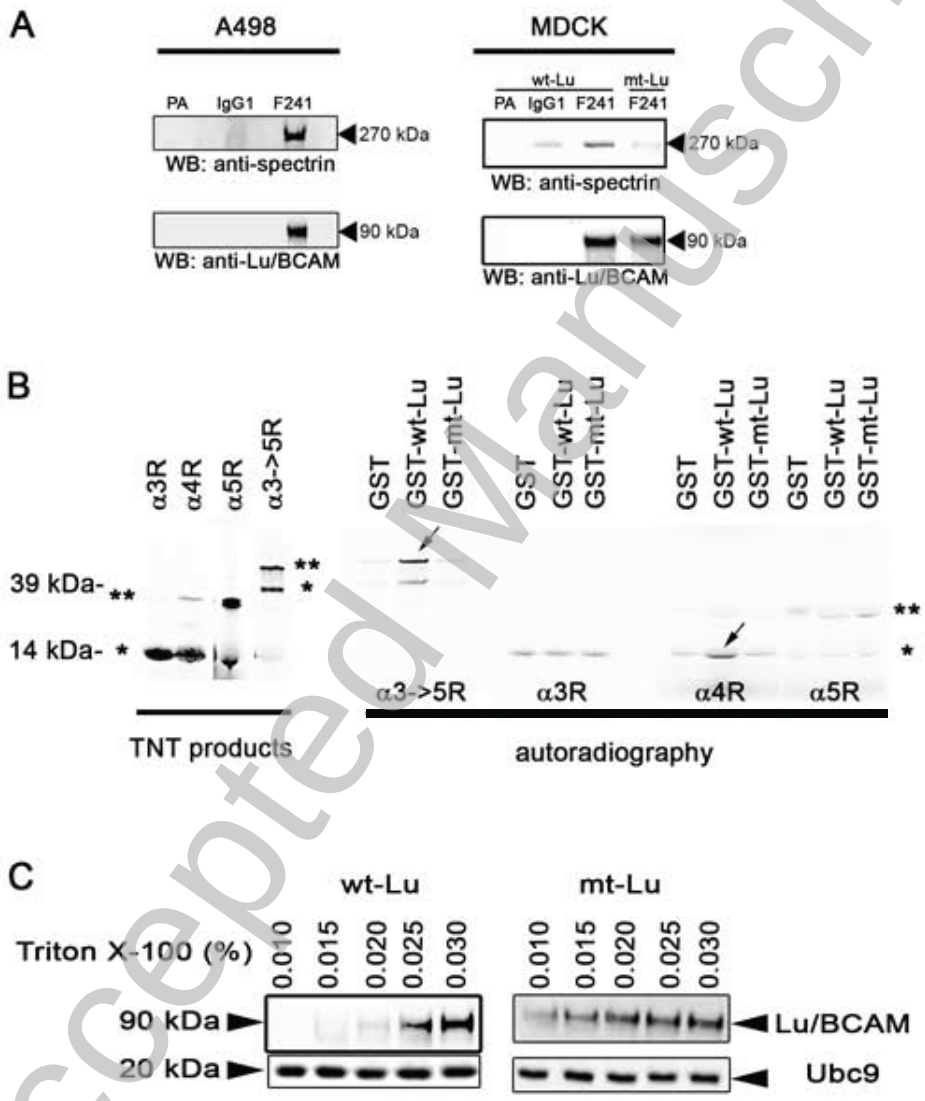

Fig.2

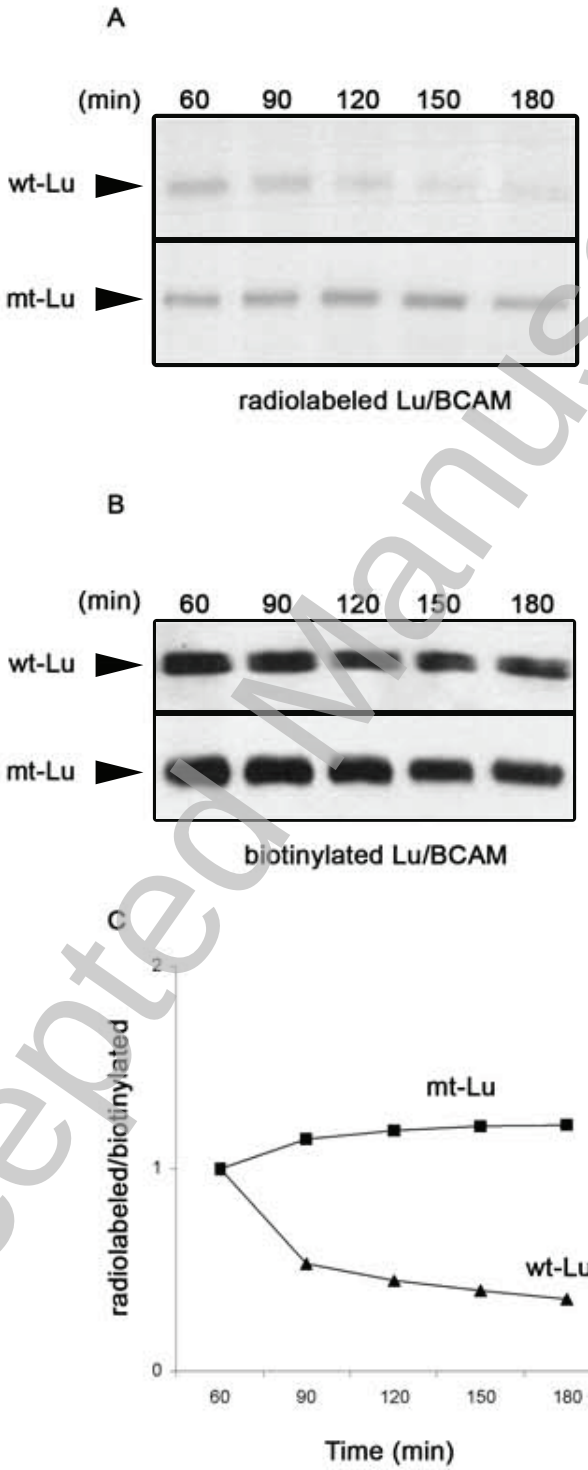

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Fig.3

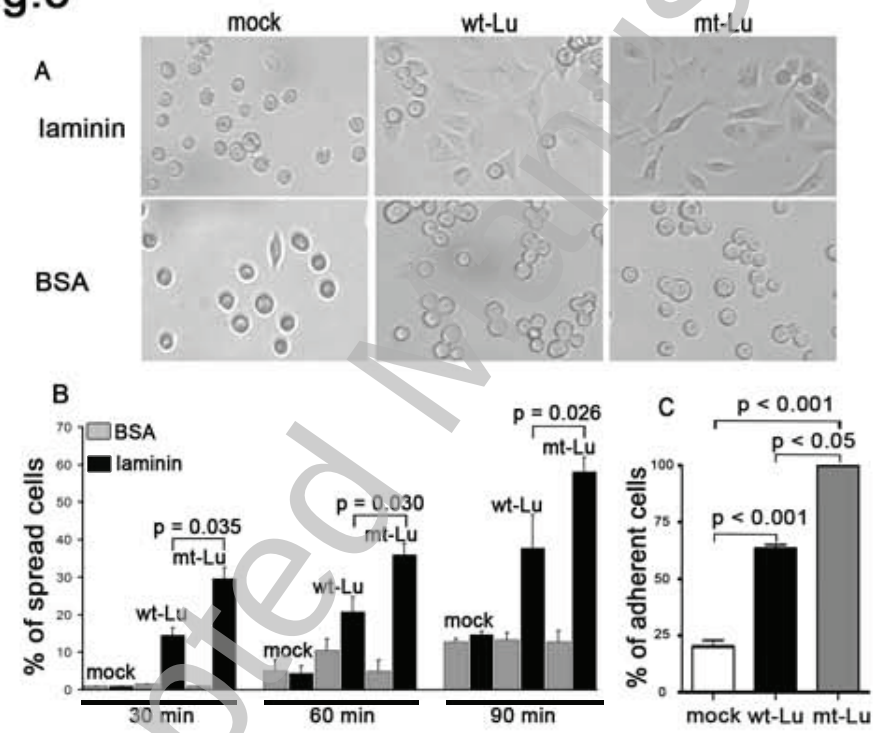

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Fig.4

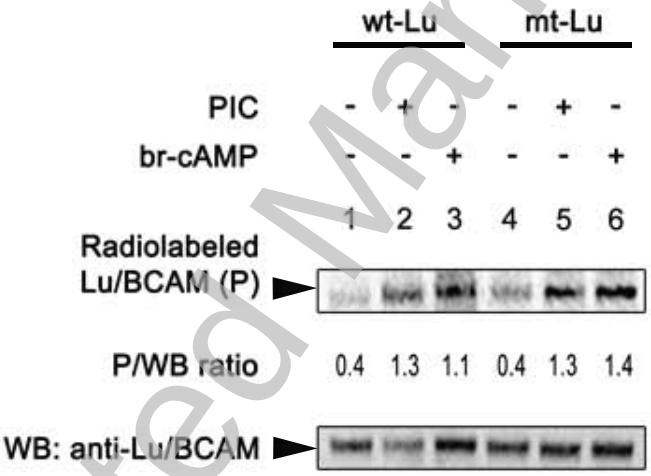


Fig.5

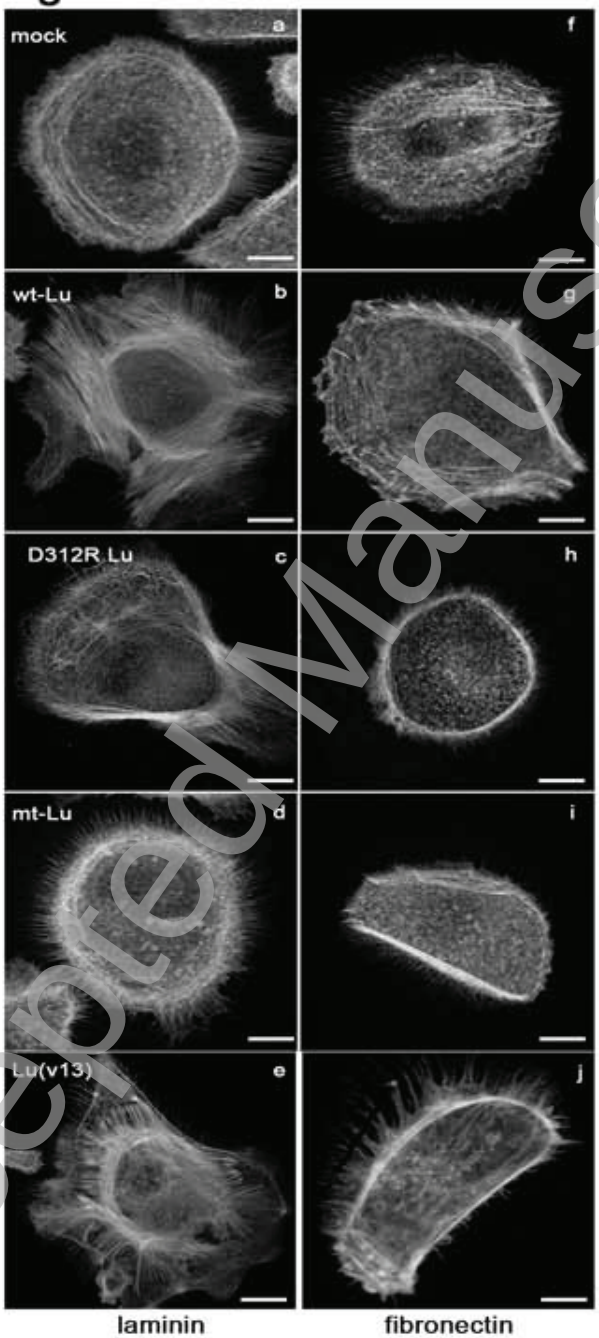
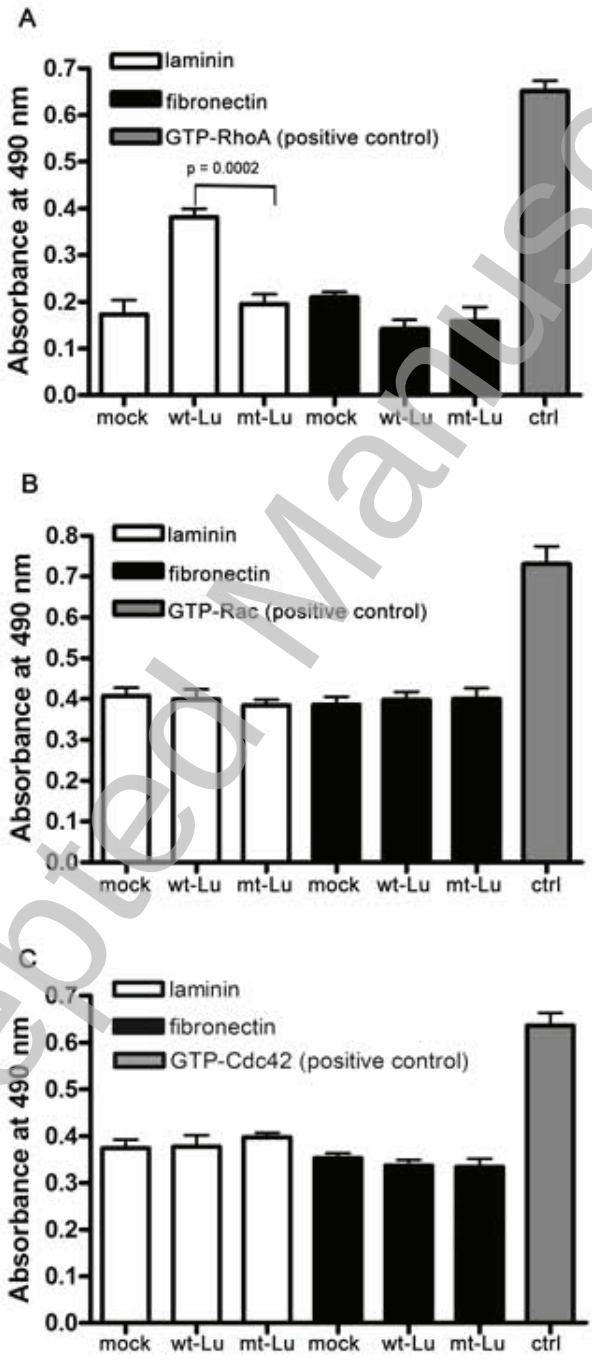


Fig.6



Biochemical Journal Immediate Publication. Published on 25 Mar 2011 as manuscript BJ20101717

Fig.7